Life history evolution, species differences and phenotypic plasticity in hemiparasitic eyebrights (Euphrasia)

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ABSTRACT

Premise of the study: Species delimitation in parasitic organisms is challenging as traits used in the identification of species are often plastic and vary depending on the host. Here, we use species from a recent radiation of generalist hemiparasitic *Euphrasia* to investigate trait variation and trait plasticity. We test whether *Euphrasia* species show reliable trait differences, investigate whether these differences correspond to life history trade-offs between growth and reproduction, and quantify plasticity in response to host species.

Methods: We perform common garden experiments to evaluate trait differences between eleven *Euphrasia* taxa grown on a common host, document phenotypic plasticity when a single *Euphrasia* species is grown on eight different hosts, and relate our observations to trait differences recorded in the wild.

Key results: *Euphrasia* exhibit variation in life history strategies; some individuals transition rapidly to flower at the expense of early season growth, while others invest in vegetative growth and delay flowering. Life history differences are present between some species, though many related taxa lack clear-cut trait differences. Species differences are further blurred by phenotypic plasticity—many traits are plastic and change with host type or between environments.

Conclusions: Phenotypic plasticity in response to host and environment confounds species delimitation in *Euphrasia*. When grown in a common garden environment it is possible to identify some morphologically distinct taxa, though others represent morphologically similar shallow segregates. Trait differences present between some species and populations demonstrates the rapid evolution of distinct life history strategies in response to local ecological conditions.

Keywords: flowering time; host range; life history evolution; parasitic plants; phenotypic plasticity, Orobanchaceae

INTRODUCTION

Parasitism is a ubiquitous feature of the natural world, with parasitic organisms present in every ecosystem and found to exploit all free-living organisms (Price, 1980; Windsor, 1998). Parasitic plants are a group of c. 4500 species of 12 separate evolutionary origins that have evolved a modified feeding organ, the haustorium, which allows them to attach to a host plant and extract nutrients and other compounds (Westwood, Yoder, and Timko, 2010; Nickrent and Musselman, 2017; Twyford, 2018). Parasitic plants are morphologically diverse and present a broad range of life history strategies and host interactions (Schneeweiss, 2006; Těšitel, Plavcová, and Cameron, 2010). Hemiparasitic plants, i.e. taxa that are parasitic but also photosynthesize, can often attach to a broad range of hosts, with the well-studied grassland parasite *Rhinanthus* found to attach to over 50 co-occurring grass and herbaceous species (Cameron, Coats, and Seel, 2006). All hemiparasitic plants are exoparasites whose leaves, stems, roots and flowers grow outside the host and only the haustorium invades and grows within the host (Twyford, 2017).

Research to date has largely focused on three aspects of life history variation in parasitic plants. Firstly, a body of work has looked to understand variation for specific traits between populations and related species. For example, work on the hemiparasite *Pedicularis* has shown how investment in male reproductive organs primarily depends on extrinsic environmental conditions (Guo, Mazer, and Du, 2010a), while seed mass is primarily determined by intrinsic factors such as plant size rather than extrinsic factors such as elevation (Guo, Mazer, and Du, 2010b). Secondly, researchers have investigated how parasite life history traits are affected by interactions with their host. In the widespread and weedy obligate holoparasite *Phelipanche ramosa*, the duration of the lifecycle differs between 14 weeks and 40 weeks depending on the host (Gibot-Leclerc et al., 2013), with evidence of local host adaptation. In hemiparasitic *Rhinanthus minor*, biomass depends on the host species and the number of haustorial connections (Rowntree et al., 2014). Finally, a number of studies have looked at life history variation between species studied in a phylogenetic context (Schneeweiss, 2006; Těšitel et al., 2010). For example, broad-scale analyses of the Rhinantheae clade in the Orobanchaceae has

shown a shift from a perennial ancestor to annuality, with correlated shifts to a reduced seed size (Těšitel et al., 2010). Despite the diversity of this research, there are still considerable gaps in our knowledge as to how life history trait variation is maintained (e.g. how common trade-offs are between life history traits), how much of this variation is genetic and how much is plastic, and which traits are the targets of natural selection.

In this study, we explore trait variation in generalist hemiparasitic eyebrights (*Euphrasia*, Orobanchaceae). *Euphrasia* is one of the largest genera of parasitic plants, and is characterized by recent transoceanic dispersal and rapid species radiations (Gussarova et al., 2008). In the United Kingdom there are 21 *Euphrasia* species, which are mostly indistinguishable at DNA barcoding loci (Wang et al., 2018), show complex morphological variation (Yeo, 1968; Metherell and Rumsey, 2018), and readily hybridize (Liebst, 2008; Stace, Preston, and Pearman, 2015). Despite shallow species differences due to postglacial divergence, *Euphrasia* species demonstrate substantial ecological divergence, with many taxa restricted to specific habitats such as coastal turf, mountain scree, heathland or open grassland. Habitat differences would be expected to exert strong selection on life history traits, and this may include selection on growth to match seasonal water availability and to exploit local hosts, or selection on flowering time in response to local competition from surrounding plants, or in response to mowing or grazing (Hellström et al., 2004).

Our research builds on a large body of experimental work, with *Euphrasia* used in common garden studies for over 125 years (Koch, 1891). The first experimental work on *Euphrasia* revealed that phenotypic differences between two related species, *E. rostkoviana* and *E. montana*, are maintained in a common garden environment (Wettstein, 1895). Experimental work in the 1960s showed the growth of various *Euphrasia* species differs depending on the host species (Wilkins, 1963; Yeo, 1964). More recent experiments using large sample sizes in common garden (Matthies, 1998; Zopfi, 1998; Lammi, Siikamäki, and Salonen, 1999; Svensson and Carlsson, 2004) or in experimental field sites (Seel and Press, 1993; Hellström et al., 2004), have shown the effect of commonly encountered hosts such as grasses and legumes on hemiparasite biomass, mineral accumulation, plant architecture and

reproductive output. Despite this extensive experimental work, studies in *Euphrasia* have yet to compare life history strategies of different species, and the extent of phenotypic plasticity in life history traits. This work is critical for improving our knowledge of hemiparasite evolution, and for understanding the nature of species differences in a taxonomically complex group. It is also unclear whether *Euphrasia* are restricted to growing on hosts such as grasses and herbaceous species, or can parasitize a broad range of taxa including novel hosts rarely encountered in the wild. To address these questions requires simultaneously investigating the growth of multiple *Euphrasia* species and multiple host species with sufficient replication to enable suitable statistical comparisons.

Here, we use a series of common garden experiments, in conjunction with field observations, to understand life history trait evolution, species differences, and phenotypic plasticity in hemiparasitic *Euphrasia*. Our first experiment assesses the morphological distinctiveness among several *Euphrasia* species and their hybrids when grown on a single host species in standardized common garden conditions. This experiment also addresses whether there is life history trait divergence among recently diverged hemiparasite species and whether these trait differences correspond to life history trade-offs. We then inspect the plasticity of a single focal *Euphrasia* population grown on many different hosts. This experiment quantifies the magnitude of trait change when *Euphrasia* are grown on different hosts. It also tests whether they are truly generalist parasites by observing their growth on a wide range of hosts as well as growing them without a host. Finally, we relate our trait observations made in a common garden to recordings made on herbarium specimens collected in the wild. This comparison will help us understand whether life history traits and species' morphological differences are consistent between the common garden and the wild. Overall, our joint observations of phenotypic variation between closely related taxa, and the extent of host-induced plasticity within a species, both in an experiment and in the wild, provide new insights into variation in life history strategies in hemiparasitic taxa.

MATERIALS AND METHODS

Experimental design and plant cultivation—We performed two common garden experiments to investigate phenotypic variation in *Euphrasia*. Both common garden experiments took place in parallel in 2016. The experiments used wild-collected open-pollinated *Euphrasia* seeds that were pooled across individuals in a population. Seeds were contributed by plant recorders as part of the 'Eye for Eyebrights' (E4E) public engagement project and as such included a scattered geographic sample across Great Britain (Appendix S1 (see the Supplementary Data with this article)). All *Euphrasia* species were identified from the herbarium specimens of field collections, and from living material grown in the glasshouse, by *Euphrasia* referee Chris Metherell. Host seeds were sourced from commercial suppliers and from field collections (Appendix S2).

Species differences experiment—We observed trait differences of twenty four populations from five *Euphrasia* species and six natural *Euphrasia* hybrids when grown on clover (*Trifolium repens*). This experiment included multiple populations of three widespread and closely related grassland species, *E. arctica*, *E. confusa* and *E. nemorosa*, and sparse population sampling of the moorland specialist *E. micrantha* (one population) and calcareous grassland specialist *E. pseudokerneri* (two populations). We chose clover as a host because it usually supports vigorous hemiparasite growth and confers high survival (Zopfi, 1998).

Phenotypic plasticity experiment—We measured traits of a focal *Euphrasia* taxon, *E. arctica*, when grown with eight potential hosts (*Arabidopsis thaliana*, *Equisetum arvense, Festuca rubra*, *Holcus lanatus*, *Marchantia polymorpha*, *Pinus sylvestris, Plantago lanceolata*, and *Trifolium repens*), and without a host. These hosts were chosen to include a broad representation of functional groups and phylogenetic diversity, with species encountered in the wild as well as novel hosts (full details in Appendix S2). The novel hosts were included to see the limits to which parasitic *Euphrasia* can associate, namely with a tree (*Pinus*), a pteridophyte that produces adventitious roots (*Equisetum*), and a liverwort that produces rhizoids (*Marchantia*).

Cultivation protocol—Reliable cultivation of *Euphrasia* can be challenging due to low seed germination, variation in time to establishment, the requirement of seed stratification, and high seedling mortality when transplanted (Yeo, 1961; Zopfi, 1998). We developed cultivation protocols that combine winter germination cues that improve germination and mimic nature, but also used highly standardized and replicated pot conditions that avoid transplanting *Euphrasia* and thus maximize survivorship. We filled 9 cm plastic pots with Melcourt Sylvamix Special growing media (Tetbury, Gloucestershire, UK) in December, placed one *Euphrasia* seed per pot, and left pots outside over winter at the Royal Botanic Garden Edinburgh (RBGE) for seeds to experience natural seed stratification. Hosts were planted in seed trays in April. *Euphrasia* plants were moved to an unheated and well-ventilated greenhouse in the spring once the cotyledons were fully expanded, and a single seedling from each host (or a $1cm^2$ clump of *Marchantia*) transplanted into the pot containing *Euphrasia*. Hosts that died within ten days of planting were replaced. Twenty or more replicates were grown for each host-parasite combination. Plants were subsequently grown to flowering with regular watering, the locations of pots randomized at weekly intervals, and foreign weed seedlings removed.

Common garden trait measurements and statistical analyses—We measured seven morphological traits at first flowering related to life history variation, indicators of plant vigor, or characters used in taxonomy. In addition to date of first flowering, we measured: corolla length, the ratio of cauline leaf length to internode length below the measured leaf ('internode ratio'), number of leaf teeth on the lower floral leaf (bract), number of nodes to flower, number of branches and plant height. All length measurements were made to the nearest millimetre, and followed Metherell and Rumsey (2018). For the phenotypic plasticity experiment, we also recorded early season growth (height six weeks after transplantation of potential host) and height at the end of season after senescence. We did not make direct observations of host attachment, as preliminary investigations revealed a fine root structure where haustoria were difficult to observe. Instead, we inferred that attachment is likely to have taken place based on observations of height, following Yeo (1964). By the end of season, *Euphrasia* that have attached to a 'good' host tended to grow tall and have elongated internodes, while *Euphrasia*

that have not attached or have attached to a 'bad' host are much smaller (see discussion for more details).

We used a combination of fixed effect and mixed models to gain insights into the differences in means and the magnitude of variability in our data. In all models, response variables were analyzed as either Gaussian (and log transformed if necessary) or Poisson. If the response variable was analyzed as Poisson, the model was checked for overdispersion and if it was overdispersed, an observation level random effect was fitted. All correlations between variables were Pearson's Correlations. Multiple correlation comparisons were corrected using Holm's correction method. Phenotypic clustering was inspected using Principal Component Analysis (PCA). All analyses were done in R version 3.4.3, with the packages lme4 (Bates et al., 2014) and MCMCglmm (Hadfield, 2010) for generalized linear mixed effects models, base R for linear models, RcmdrMisc for correlations (Fox, 2018) and ggplot2 for data visualisation (Wickham, 2016). MCMCglmm models were run for a minimum of 70,000 iterations using either inverse Wishart or parameter expanded priors with a minimum burn in period of 30,000 iterations. Model convergence was assessed visually by plotting the posterior distributions and Markov chains.

In the species differences experiment, species of *Euphrasia* was fitted as a fixed effect and population of *Euphrasia* was treated as a random effect. We excluded hybrids from these analyses because we were interested in testing differences between species. In the case of height and cauline:internode ratio the traits were log transformed. Likelihood Ratio Tests calculated the overall significance of species, where this was not possible, Deviance Information Criteria were used to test better model fit. We calculated proportion variance explained by population of *Euphrasia* (after accounting for fixed effects) by dividing the population random effect variance by the total variance in the model. Tukey post hoc tests were performed on each pairwise comparison of *Euphrasia* species and adjusted p-values calculated, using Tukey HSD (Honest Significant Difference) correcting for family-wise error rate in the emmeans R package (Lenth, 2019). For the phenotypic plasticity experiment, host species was fitted as a fixed effect. The models were re-levelled so that 'no host' was the baseline. Analysis of variance was used to determine overall significance of host species. Tukey post hoc tests were then

performed on each pairwise comparison of host species, with adjusted p-values calculated in base R and the multcomp package (Hothorn, Bretz and Westfall, 2008).

Trait variation in the wild—We tested how phenotypes in the experiments related to those in nature by comparing results from the species differences experiment to phenotypic measurements made on herbarium specimens of the same population sampled in the wild. Three individuals were measured from each collection sheet for a given population for each trait. Pressed plants submitted by collectors varied in quality and therefore we were unable to measure the height of these plants, nor was it possible to infer date of first flowering. We analyzed the data using generalized linear mixed effect models with where individuals were grown (i.e. common garden or wild-collected) as a fixed effect, with each of five traits as the response variable. We treated species and population of *Euphrasia* as random effects to understand the relative contributions of each to the overall variability in a given trait. Response variables that were considered count data were analysed with a Poisson distribution, in all other cases a Gaussian distribution was used. R-values were calculated using Pearson's correlations of the population level means between the common garden and the wild samples.

RESULTS

Species differences—Our species differences experiment revealed extensive morphological trait variation across *Euphrasia* species when compared at first flowering. From the 222 *Euphrasia* individuals that survived to flower on their clover host, the greatest variation was seen in number of branches (9-fold difference between species), internode ratio (2.7-fold) and height (2.5-fold), while traits such as node to flower (1.6-fold) and corolla length (1.6-fold) proved less variable (Fig. 1a-d; Appendix S3). A large degree of this variation was separated by species and by population (Table 1). The species with the most distinct life-history strategy was *E. micrantha*, which flowered from a low node on the plant (8.3 ± 0.2 nodes) while it was short (70 ± 8 mm; Appendix S3). It also formed a partly distinct cluster in the PCA analysis (Appendix S4). *E. pseudokerneri* was relatively distinct, flowered once it has grown tall (176 ± 16 mm) and from a high node on the plant (13.2 ± 0.4 nodes),

but showed little separation in the PCA analysis. The morphologically similar *E. arctica*, *E. confusa* and *E. nemorosa* differed for some traits, with *E. nemorosa* initiating flowering 14 days later and from 3.3 nodes higher than *E. arctica*, but overlapped in many other traits and in overall multi-trait phenotype (Appendix S4, S5). Despite species being a significant factor in the models, and some notable differences in specific traits, there were few significant pairwise Tukey comparisons due to substantial within species variation (Appendix S6). Of the seven significant pairwise trait differences three were for node to flower and three for number of leaf teeth, with four of the seven significant comparisons involving *E. micrantha*. In most cases hybrids combined morphological characters of their parental progenitors, for example hybrids involving *E. nemorosa* flowered later in the season and initiated flowering from a higher node than *E. arctica* hybrids (Fig. 1a-d).

Correlation analyses across species revealed clear suites of traits that are related. Significant correlations were found between 12 of the 21 pairwise comparisons after correcting for multiple tests, with 5 of these correlations with an $R > 0.6$ (Table 2a). Plants flowering at a late node are more likely to be tall, more highly branched, as well as having many teeth on the lower floral leaf. The relationship of traits is also supported in the PCA analysis, with many traits contributing to multiple principal components (Appendix S5). Height and flowering node related traits are largely uncorrelated with cauline internode-leaf ratio and corolla length.

Phenotypic plasticity—Our phenotypic plasticity experiment showed substantial morphological variation across 194 *E. arctica* plants grown with 8 different potential host species, and the 22 plants grown without a host. Plants growing on clover transitioned to flower quickly (189.8 ± 2.0 Julian days), grew tall by the time of first flowering (39 ± 3 mm), and produced large flowers (7.4 ± 0.2 mm; Fig. 1e-h, Appendix S7). This contrasts with *Euphrasia* with no host, which flowered on average 52 days later (241.3 ± 7.9 Julian days), were extremely short at first flowering (11 ± 1 mm), and produced small flowers (5.3 ± 0.2 mm). *E. arctica* grown on *Arabidopsis*, *Equisetum*, *Festuca*, *Holcus* or *Plantago* were all statistically significantly different to no host for at least one trait (Tukey comparisons, $P < 0.05$), while *E. arctica* on *Marchantia* or *Pinus* were not significantly different from no host for any trait ($P > 0.05$; Appendix S8). While the overall effect of host was significant for all

traits except nodes to flower (Appendix S9 – S11), three traits showed relatively little plasticity, with few statistically significant pairwise Tukey comparisons for nodes to flower (0 significant comparisons), number of leaf teeth (3) and internode length (4), while the other three traits showed many pairwise differences (days to flower, 21 significant comparisons; height, 16; corolla length, 12; Appendix S8). Our comparison of growth across host treatments measured through the year showed that height at the end of the season is weakly predicted from height 6-weeks after introducing a host ($R = 0.47$), but strongly correlated with height at first flowering ($R = 0.82$, Appendix S12). Plants that flowered early were more likely to grow larger by the end of season ($R = -0.55$) and become more highly branched ($R = -0.57$, Appendix S12).

Across host treatments, there was a significant negative correlation between Julian days to flower and most other traits (Table 2b). We find that late flowering individuals are likely to be smaller at first flowering, have fewer branches, have leaves with fewer teeth, and have smaller flowers. While these traits were strongly correlated, there were substantial differences in the magnitude of response. For example, days to flower differed considerably depending on host, with a 3.8-fold greater difference than seen between means of different *Euphrasia* species grown on the same host (Fig. 1d & 1h). In contrast corolla length and node to flower proved less variable depending on host, with a 1.4-fold and 1.2-fold change between means, respectively.

Variation in the wild—The comparison between the species differences common garden experiment and wild-collected herbarium specimens revealed population means of a single trait, nodes to flower, are strongly correlated ($R = 0.79$) and trait values are not significantly different ($p_{MCMC} = 0.71$) between environments (Fig. 2; Appendix S13; S14). All other traits did differ significantly between environments ($p_{MCMC} < 0.05$), with *Euphrasia* plants in the common garden having corollas on average 1.4 mm longer, with 0.2 more teeth on the lower floral leaves, an increase in cauline:internode ratio of 1.0 mm, and 4 more pairs of branches. Despite these differences, there were correlations between the common garden and the wild-collected specimens for corolla length ($R = 0.93$, $p_{MCMC} < 0.001$), cauline internode:leaf length ratio ($R = 0.65$, $p_{MCMC} < 0.001$) and number of branches ($R = 0.29$, $p_{MCMC} < 0.001$), but not for number of leaf teeth ($R = 0.07$, $p_{MCMC} = 0.034$).

DISCUSSION

Our study sheds light on species differences, life history evolution and phenotypic plasticity of the generalist parasitic plant *Euphrasia*. We find different life history strategies between recently diverged species, with some species rapidly transitioning to flower at the expense of growth-related traits, while others delay flowering and invest in early-season vegetative growth. However, many traits are phenotypically plastic and change in response to the host. While plants in benign common garden conditions grew vigorously, the correlation between life history traits in a common garden and in the wild suggests our experimental observations are indicative of patterns observed in nature. Morphological differences between species in the common garden also suggest that the currently delimited *Euphrasia* species are, at least in part, distinct. Overall our study highlights the value in integrating trait data from multiple common garden experiments and field collections to study life history strategies in parasitic plants, and demonstrates the rapid evolution of life history differences in a postglacial radiation of hemiparasites.

Life history variation in a generalist hemiparasitic plant—Our study finds evidence for different life history strategies in British *Euphrasia*. *E. arctica*, *E. micrantha* and hybrids such as *E. arctica* x *E. confusa*, transition rapidly to flower, flower while they are short, and produce their first flower from a low node on the plant. This contrasts with *E. pseudokerneri*, *E. nemorosa* and hybrids involving *E. nemorosa* that delay flowering until later in the season, grow tall before flowering, and produce their first flower from a late node on the main axis. These different life history strategies correspond to the known ecology of these species, with *E. nemorosa* flowering late in tall mixed grassland, while *E. micrantha* flowers early in patchy heathland (Metherell and Rumsey, 2018). While species show some general differences in life history strategies, there is also significant variation between populations within species. A relationship between internode number and habitat has previously been observed within *Euphrasia* species, with populations of *E. rostkoviana* in Sweden flowering at a lower node in a common garden if they have been collected from intensely grazed

299 pasture (Zopfi, 1998). Overall, these observations within and between species are consistent with the
300 classic life history trade-off between growth and reproduction (Stearns, 1992; Roff, 2002). For
301 *Euphrasia* growing in the wild, early reproduction allows the plants to reliably complete their
302 lifecycle before summer competition, herbivory, mowing, summer drought and other seasonal abiotic
303 and biotic stresses. However, early flowering involves reproducing at the expense of early season
304 growth and at a time when the resource budget may be constrained by relatively few haustorial
305 connections. These trait trade-offs pose an interesting comparison to the well-studied *Mimulus*
306 *guttatus* (*syn. Erythranthe guttata*), a non-parasitic relative in the Lamiales that shares the same basic
307 plant architecture. In *M. guttatus* multiple traits related to growth and reproduction are correlated both
308 within and between populations, due to genetic trade-offs between time to flower and fecundity
309 (Mojica et al., 2012; Friedman et al., 2015). In *Euphrasia* the genetics underpinning this life history
310 trade-off have yet to be characterized, and may be a consequence of multiple independent loci or
311 trade-offs at individual loci (Hall, Lowry, and Willis, 2010).

312 While much life history variation is captured by differences in time to flower and growth-related
313 traits, we also see evidence for flower size representing a separate axis of variation across *Euphrasia*
314 species. In our common garden *E. micrantha* has small corollas, while *E. arctica* and *E. nemorosa*
315 have larger corollas, and corolla size is not strongly correlated with other traits. *Euphrasia* species are
316 well-known to have flower size variation, with a continuum between small flowered species that are
317 highly selfing (e.g. *E. micrantha*, corolla size = 4.5 - 6.5mm, inbreeding coefficient $F_{IS} > 0.88$, Stone,
318 2013), and large flowered species that are highly outcrossing (e.g. *E. rostkoviana* flower size 8 -
319 12mm, $F_{IS} = 0.17 - 0.25$ French et al., 2005). Such wide variation in outcrossing rate has been
320 documented in species of *Datura* (Motten and Stone, 2000), *Mimulus* (Karron et al., 1997) and
321 *Nicotiana* (Breese, 1959). Small flowers have shorter anther-stigma separation and thus increased
322 potential for autogamous selfing (Karron et al., 1997), while also having reduced attractiveness to
323 pollinators and thus receiving less outcross pollen (Mitchell et al., 2004). In addition to differences in
324 corolla size between *Euphrasia* species, corolla size also shows a change of up to two millimetres in
325 response to host species. This change in flower size is of a magnitude that may potentially affect the

mating system (Luo and Widmer, 2013), and suggests host species represents a previously unaccounted factor affecting the mating system of parasitic plants.

Our comparisons of *Euphrasia* species in a common garden also sheds light on the distinctiveness of these recently diverged species, and can be used to refine the suite of traits that are reliable in telling *Euphrasia* species apart. *Euphrasia* is a taxonomically complex plant genus, with the 21 currently described British species presenting complex and often overlapping morphological variation (French et al., 2008; Metherell and Rumsey, 2018; Wang et al., 2018). Our study suggests varying degrees of morphological distinctiveness of *Euphrasia* species. We see *E. micrantha* is morphologically distinct in the common garden, and *E. pseudokerneri* somewhat distinct, while the closely related species *E. arctica*, *E. confusa* and *E. nemorosa* differ in life history traits such as nodes to flower, but overlap in many other traits and are not clearly separated in the PCA. The morphological trait differences between species observed under standardised conditions are correlated with values from field-collected herbarium specimens where plants have associated with diverse hosts, been exposed to different ecological conditions, and were collected at different life-stages. This suggests that our common garden results generalize to observations in nature. However, our study is likely to overestimate the distinctiveness of taxa by only including a subset of UK species and by choosing populations that could be identified to species-level in the field. We suspect adaptive divergence between closely related *E. arctica*, *E. confusa* and *E. nemorosa* is a consequence of differential natural selection for local ecological conditions such as soil water availability or mowing. Selection appears to be operating at a fine spatial scale, with significant life history trait differences evident between populations within species. *Euphrasia* taxa may be genetically cohesive, either showing genome-wide divergence or divergence in genomic regions underlying life history differences (Twyford and Friedman, 2015), or alternatively these taxa may be polytopic and not genetically cohesive (Hollingsworth, Neaves, and Twyford, 2017). Genomic sequencing of natural populations will help resolve the nature of species differences in *Euphrasia*.

Phenotypic plasticity in response to host—Our phenotypic plasticity experiment shows *Euphrasia* are affected by growing with a range of different hosts. Specifically, *E. arctica* with a host such as

353 clover rapidly transitions to flowering. At the other extreme, *Euphrasia* grown without a host are
354 small and flower late. These differences in growth are established early in the season, and early
355 flowering plants go on to grow the tallest and are more highly branched. Most other hosts result in a
356 continuum of *Euphrasia* phenotypes between these extremes. Two surprising results were that *E.*
357 *arctica* parasitizing *Arabidopsis* grew relatively tall despite the host senescing early in the growth
358 season, and that *Euphrasia* growing with *Equisetum* performed similarly to when grown on the
359 commonly encountered grass *Holcus lanatus*. This suggests attachment to *Equisetum*, which would
360 need to be confirmed by excavating root systems and observing haustoria, or indirect benefits without
361 attachment through association with *Equisetum* fungal symbionts (Bouwmeester et al., 2007). Less
362 surprising was the poor growth of *E. arctica* with *Pinus*. However, an association between
363 *Melampyrum pratense* and *Pinus sylves*tris suggests at least some hemiparasitic Orobanchaceae
364 benefit from attachment to woody host species, or interactions with their associated ectomycorrhizal
365 fungi (Salonen et al., 2000).

366 The diverse effects of host on parasite growth are complex, but the variation we see in our
367 experiments may be attributed to host root architecture, germination time and resource availability, as
368 well as the presence of mechanisms to defend against parasite attack, such as cell wall thickening,
369 localized host dieback, and chemical defence (Cameron, Coats, and Seel, 2006; Twyford, 2018).
370 While *Euphrasia* is generally thought to have low reliance on host resources, deriving only ~30% of
371 carbon heterotrophically (Těšitel, Plavcová, and Cameron, 2010), at least under our experimental
372 conditions *Euphrasia* only produced multiple flowers on certain hosts. Overall our results point to *E.*
373 *arctica* being a true generalist hemiparasite, but one where vigorous growth is only observed with a
374 subset of potential hosts.

375 In terms of specific traits, only three pairs of trait correlations show consistent correlation coefficients
376 in both *Euphrasia* common garden experiments (between height, number of branches and leaf teeth),
377 with other correlations between species breaking down when *Euphrasia* are grown on different hosts.
378 The most notable plasticity is seen in flowering time, with plants on clover rapidly transitioning to
379 flower within ~100 days of germination, while plants with a more typical host (e.g. *Holcus lanatus*)

flower a month later. Phenotypic plasticity in flowering time in response to resource availability is well documented in many plant groups, particularly *Arabidopsis* (e.g. Zhang and Lechowicz, 1994), but has received less attention in studies of parasitic plants, which are more likely to look at growth-related traits such as biomass (Ahonen, Puustinen, and Mutikainen, 2005; Matthies, 2017). However, date of first flowering has been shown to differ by up to 10 weeks in populations of *Rhinanthus glacialis* across Switzerland (Zopfi, 1995). Overall, we expect date of first flowering to be critical for the life time reproductive success of parasitic plants in the wild.

In contrast to traits showing extensive plasticity, we also see evidence of developmental constraint in number of nodes to flower. For *E. arctica*, this trait showed the least plasticity with different hosts, is consistent between populations within species, and between the common garden and the field. This suggests that the developmental event of transitioning to flower is genetically determined, with changes in flowering time altered by plasticity in internode length and not nodes to flower. This may explain why nodes to flower is such an important diagnostic trait for species identification in *Euphrasia* and related species in the Rhinantheae (Jonstrup, Hedrén, and Andersson, 2016). Despite nodes to flower changing little in response to host species, our overall impression is that *Euphrasia* show considerable plasticity and little developmental constraint in many aspects of growth. In particular, differences between individuals on a given host also suggests other sources of variation, such as genetic background in host and parasite, as well as the timing of attachment, may be crucial in determining performance.

CONCLUSIONS

Despite over a century of experimental studies in parasitic plants, our understanding of the evolution of life history strategies in these diverse organisms is extremely limited. Our results with *Euphrasia* provide strong support for the rapid evolution of distinct life history strategies in response to local ecological conditions, with phenotypic plasticity further altering plant growth in response to host availability. We anticipate that future studies that test life time reproductive success of many parasitic

plant species grown on many different host species will give further insight into the complex nature of host-parasite interactions.

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Author Contributions

A.D.T conceived and designed the research. A.D.T., N.F. and E. L. Y.W. carried out the experiments. C.M. identified the plants. A.D.T. and M.B. analysed the data. A.D.T. and M.B. wrote the manuscript. All authors read and approved the manuscript.

Data Availability Statement

Phenotypic data from both common garden experiments and from herbarium collections, as well as the R scripts used for data analysis, are deposited in Dryad (doi.org/10.5061/dryad.cfxpnvx2d). Source code and data are also deposited on Github: https://github.com/Euphrasiologist/phenotypic_plasticity_euphrasia

Supporting information

Additional Supporting Information may be found online in the supporting information section at the end of the article.

Appendix S1. Collection details for *Euphrasia* species used in the common garden experiments.

Appendix S2. Species and collection details for hosts used in the phenotypic plasticity common garden experiment.

Appendix S3. Summary of trait values for many *Euphrasia* species and hybrids grown on a clover host. Values are mean +/- one standard error. Length measurements are in mm.

Appendix S4. Principal component analysis of morphological variation of *Euphrasia* in a common garden for (a) five species and six hybrids grown with a clover host, (b) five species grown with a clover host, (c) *E. arctica* with nine host treatments. Points represent individuals, and ellipses represent the standard error of the (weighted) average of scores.

Appendix S5. Factor loadings for the principal component analyses of (a) five species and six hybrids, (b) five species, (c) *Euphrasia arctica* with nine host treatments.

Appendix S6. Comparison of *Euphrasia* species differences of plants grown with clover in a common garden experiment. Tukey comparisons are presented between each pair of species, with significant comparisons shown in bold. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

Appendix S7. Summary of trait values for *Euphrasia arctica* grown on many different hosts. Values are mean +/- one standard error. Length measurements are in mm.

Appendix S8. Comparison of *E. arctica* traits in the phenotypic plasticity common garden experiment. Tukey comparisons are presented between *E. arctica* traits with two different host treatments. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

Appendix S9. Analysis of deviance for each trait in the phenotypic plasticity experiment with *E. arctica* grown with many different hosts, assuming a Poisson distribution. For each model, we report the change in degrees of freedom (Df), deviance, residual degrees of freedom, residual deviance, and p-value generated from the Chi-squared distribution. Factor host is compared to the intercept model where no factors are fitted.

458 **Appendix S10.** ANOVAs for traits measured in the phenotypic plasticity experiment with E. arctica
459 grown with many different hosts, assuming Gaussian distributed residuals. For each model, we report
460 the degrees of freedom (Df), sums of squares (SS), mean squares (MS), F-statistic, and P-value.

461 **Appendix S11.** Summary of generalised linear models for the phenotypic plasticity experiment with
462 *Euphrasia arctica* grown on many hosts in a common garden. All models compare *E. arctica* grown
463 with a particular host to the intercept of no host. Generalised linear models assuming Poisson
464 residuals with log link function were used in Julian days to flower, nodes to flower and number of leaf
465 teeth, while all others assumed Gaussian residuals. The model coefficient is reported with standard
466 error in brackets. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

467 **Appendix S12.** Relationship between growth-related traits and end of season height for *E. arctica*
468 grown with nine different host treatments. (a) Height at first flowering, (b) height 6-weeks after
469 germination, (c) Julian days to flower, (d) number of branches. Length measurements are reported in
470 mm.

471 **Appendix S13.** Comparison between trait values for wild-collected herbarium specimens and
472 common garden plants of diverse *Euphrasia* species for (a) nodes to flower, (b) corolla length (mm),
473 (c) number of leaf teeth, (d) internode ratio. Points are for *Euphrasia* population means, with bars
474 representing the standard error of measurements.

475 **Appendix S14.** Model output from MCMCglmm comparing traits for the wild collected *Euphrasia*
476 specimens to the baseline of the common garden data (Intercept). The posterior means are reported
477 along with the lower and upper 95% credible intervals, as well as the p-value (pMCMC) for the effect.

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Tables

Table 1. Summary of generalized linear mixed effects models for *Euphrasia* trait values measured in a common garden environment. Table summarizes model outputs for five *Euphrasia* species grown with clover in the species differences experiment, and *E. arctica* grown with eight hosts plus no host treatment in the phenotypic plasticity experiment. For the phenotypic plasticity experiment, we report model outputs with all potential hosts, as well as models excluding *Pinus* and *Marchantia* where there was no evidence of attachment or interactions (reported in square brackets). The percentage variance explained by random effects are reported in brackets along with the 95% credibility interval. *** P < 0.001, ** P < 0.01, * P < 0.05

	Species differences		Phenotypic plasticity
	Species	Population	Host
Branches	$DIC_{full} = 676.08$; $DIC_{-spp} = 679.48$	$DIC_{full} = 676.08$; $DIC_{-pop} = 714.87$ (25.9%, 13.4 – 57.4%)	NA
Corolla length	$\chi^2(4) = 11.91^{*}$	$\chi^2(1) = 41.38^{***}$ (54.1%, 24.4 – 69.2%)	$(F_{8,173} = 9.85)^{***}$ [$(F_{6,157} = 11.38)^{***}$]
Height	$\chi^2(4) = 11.67^{*}$	$\chi^2(1) = 57.13^{***}$ (61.2%, 35.0 – 79.7%)	$(F_{8,185} = 23.14)^{***}$ [$(F_{6,164} = 24.39)^{***}$]
Internode ratio	$\chi^2(4) = 13.00^{*}$	$\chi^2(1) = 34.38^{***}$	$(F_{8,184} = 3.36)^{**}$ [$(F_{6,163} = 4.11)^{***}$]
Julian days to flower	$\chi^2(3) = 2.26$	$\chi^2(1) = 1.42$ E-14 (58.7%, 28.6 – 80.9%)	$\chi^2(8) = 192.39^{***}$ [$\chi^2(6) = 141.67^{***}$]
Node to flower	$\chi^2(4) = 15.42^{**}$	$\chi^2(1) = 2.87$	$\chi^2(8) = 5.02$

		(14.1%, 1.0 – 33.5%)	[$\chi^2(6) = 3.04$]
Number of leaf teeth	$\chi^2(3) = 12.45^{**}$	$\chi^2(1) = 0.0059$ (0.12%, 2.8% - 23%)	$\chi^2(8) = 26.79^{***}$ [$\chi^2(6) = 17.04$**]

Note: Models for number of branches were implemented with a different statistical approach in MCMCglmm, with models compared using Deviance Information Criterion (DIC; see methods).

Table 2. Pearson's correlation coefficients for seven phenotypic traits measured in a common garden experiment for (a) Five *Euphrasia* species and 6 hybrids, (b) *Euphrasia arctica* grown with 8 hosts and without a host. *** P < 0.001, ** P < 0.01, * P < 0.05. Asymptotic p-values values are reported from the Hmisc package in R using the rcorr() function.

(a) Species differences experiment

	Corolla length (mm)	Height (mm)	Internode ratio	Julian days to flower	Leaf teeth	Nodes to flower
Branches	0.260 **	0.609***	-0.116	0.057	0.658***	0.775***
Corolla length (mm)		0.319***	-0.161	-0.127	0.197 *	0.049
Height (mm)			0.246**	0.292*	0.563***	0.628***
Internode ratio				0.204	-0.120	0.076
Julian days to flower					0.053	0.249*
Leaf teeth						0.651***

(b) Phenotypic plasticity experiment						
	Corolla length (mm)	Height (mm)	Internode ratio	Julian days to flower	Leaf teeth	Nodes to flower
Branches	0.524***	0.834***	-0.299***	-0.572***	0.694***	-0.572**
Corolla length (mm)		0.503***	0.098	-0.406***	0.536***	-0.166
Height (mm)			0.477***	-0.481***	0.692***	-0.186
Internode ratio				-0.034	0.168	-0.009
Julian days to flower					-0.691***	0.530***
Leaf teeth						-0.239**

632

633

634 **Figure legends**

635 **Figure 1.** Trait variation in a common garden experiment of diverse *Euphrasia* species and hybrids
636 grown on clover (A-D); *Euphrasia arctica* grown on many different hosts (E-H). The edges of the
637 boxplots show the first and third quartiles, the solid lines the median, the whiskers the highest and
638 lowest values within 1.5-fold of the inter-quartile range and the jittered dots each individual
639 measurement. Length measurements were recorded in millimetres.

640 **Figure 2.** Relationship between morphological trait measurements made in the common garden and
641 on wild-collected herbarium specimens for diverse *Euphrasia* species. Points are *Euphrasia*
642 population means, with bars representing the standard error of measurements. The line of best fit was
643 calculated using coefficients from linear regression models on the means of each *Euphrasia*
644 population. Length measurements are reported in mm. For an alternative representation of pairwise
645 comparisons see Appendix S13.